Essential role of coenzyme A in pyruvate dehydrogenase kinase activity

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Received 16 September 1982

The rate of phosphorylation and concomitant inactivation of purified pig heart muscle pyruvate dehydrogenase complex by intrinsic kinase (EC 2.7.1.99) is markedly accelerated by the addition of coenzyme A to the incubation medium, showing a half-maximum effect at 1.8 μM. The pantetheine moiety is the effective part of the coenzyme A molecule. The free thiol group is prerequisite for the stimulatory action, acetyl-CoA, benzoyl-CoA or CoAS-SCoA being ineffectual. The thiol's specificity is evidenced by showing that dithiothreitol, 2-mercaptoethanol or glutathione up to 5 mM failed to replace coenzyme A. The possibility is considered that coenzyme A might act as a physiological modifier of pyruvate dehydrogenase kinase activity.

Pyruvate dehydrogenase kinase *Phosphorylation* *Coenzyme A* *Heart muscle*

1. INTRODUCTION

Recent work on highly purified pyruvate dehydrogenase complex (PDH) from bovine kidney suggests that protein thiol–disulfide exchange is involved in the regulation of PDH kinase (EC 2.7.1.99) activity [1]. Disulfides, first of all, 5,5′-dithiobis (2-nitrobenzoate) (DTNB) markedly lowered the activity of the enzyme, and this was reversed by glutathione (GSH) and other thiols [1]. Neither the compound(s) that may be physiologically responsible for the postulated modification, nor the nature of the thiol group(s) are known. A possible candidate may be represented by coenzyme A (CoASH), reported to specifically restore endogenous PDH kinase activity lowered upon dialysis [2]. With respect to the findings in [1] it appears timely to present more detailed evidence in support of the view that CoASH might act as a physiological modifier of kinase activity.

2. MATERIALS AND METHODS

Coenzyme A, dephospho-CoASH, acetyl-CoA, ATP, NAD^+, glutathione and pyruvate were bought from Boehringer (Mannheim). Benzoyl-CoA was prepared as in [3]. CoAS-SCoA was prepared by incubation of 50 μl 50 mM CoASH together with 50 μl 0.1 M Na_2CO_3, 200 μl 30 mM Tris–HCl buffer (pH 8.6) and 20 μl 32 μM $CuSO_4$ at 25°C. After 2 h, >90% of the initially titratable thiol content [4] had disappeared. [γ-^{32}P]ATP was purchased from the Radiochemical Centre (Amersham). PDH was purified from pig heart muscle as in [5]. PDH-phosphatase was prepared as in [6]. PDH activity was assayed spectrophotometrically by recording NAD^+ reduction with pyruvate as the substrate [7]. One unit corresponds to the formation of 1 μmol NADH/min at 25°C under the conditions detailed in [7]. Incorporation of ^{32}P from [γ-^{32}P]ATP into protein was measured as in [8].

3. RESULTS

The rate of inactivation of purified PDH complex preparations by ATP can display considerable variation. Thus, preparations after dialysis were usually found to be inactivated at a markedly lower rate than before dialysis, indicating a loss in PDH kinase activity by the dialysis step. This could largely be restored by the addition of CoASH to the incubation mixture used for PDH inactivation (fig.1). Concomitantly with the rate of inactivation CoASH increased that of protein phosphorylation (fig.1). Recovery of the original

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00145793/82/0000–0000/$2.75

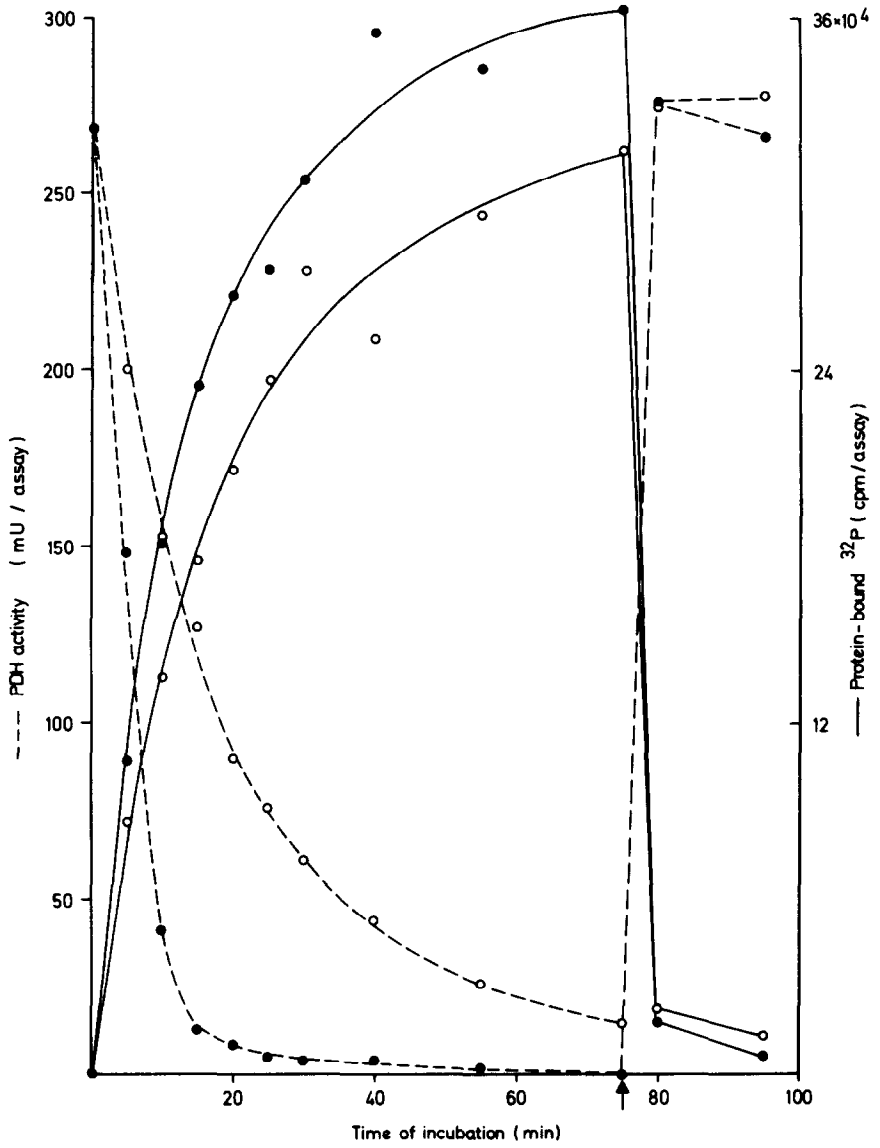

Fig.1. Effect of CoASH on the rate of PDH phosphorylation and inactivation. The reaction mixture containing 150 μl PDH dialyzed against 50 mM histidine–HCl buffer (pH 6.9), 20 μl 1.6 mM 2-mercaptoethanol, 25 μl 0.32 mM [γ-^{32}P]ATP, 25 μl 1.3 mM $MgCl_2$ and 20 μl 0.36 mM CoASH in the above histidine buffer (•) or buffer only (○) was incubated at 25°C for the indicated times before 10 μl aliquots were assayed for PDH activity and ^{32}P-incorporation. At the time indicated by the arrow [Mg^{2+}] was increased to 10 mM and purified PDH-phosphatase was added.

PDH activity and dephosphorylation of the enzyme was achieved by the subsequent addition of PDH-phosphatase and 10 mM Mg^{2+}, indicating that CoASH had caused no irreversible alteration of PDH activity. Moreover, CoASH remained ineffectual on PDH activity when the incubation mixture was devoid of ATP (not shown). In the presence of 25–100 μM ATP the CoASH effect (fig.2) did not appear to depend essentially on this nucleotide. As the K_M for ATP of PDH kinase has

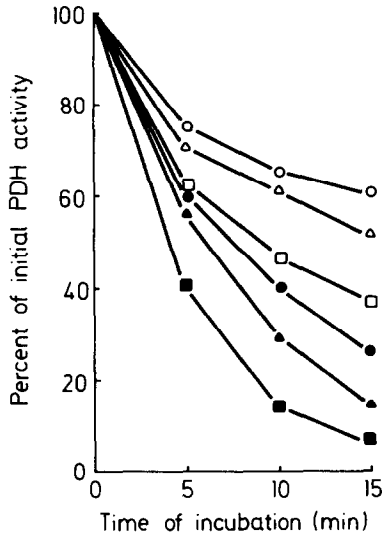

Fig.2. Effect of CoASH on PDH inactivation at various ATP concentrations. The incubation mixture contained 30 μl PDH dialyzed against 50 mM histidine–HCl buffer (pH 6.9), 5 μl 0.3 mM CoASH in the same buffer (filled symbols) or buffer only (open symbols), 10 μl 1 mM $MgCl_2$ and 5 μl ATP solution to yield a final concentration of 25 μM (circles), 50 μM (triangles) and 100 μM (squares), respectively. Incubation was carried out for the indicated times before 10 μl aliquots were analyzed for PDH activity which initially amounted to 36 mU/assay.

been determined to be ~20 μM, CoASH does not seem to act by increasing the affinity of this enzyme for ATP.

Fig.3 illustrates the relationship between the stimulation of PDH inactivation and CoASH concentration. From the double-reciprocal plot of the data (fig.3, inset) it appears that the CoASH effect was half-maximal at 1.8 μM. Experiments performed with the aim to determine the essential part of the CoASH molecule revealed that the presence of the pantetheine moiety is essential. This is indicated by the finding that adenosine or 5′-AMP were ineffective whereas dephospho-CoASH, pantetheine or *N*-acetylcysteamine were stimulatory. Compared to CoASH or the equivalent dephospho-CoASH, however, much higher concentrations of pantetheine and *N*-acetylcysteamine had to be employed, the half-maximal doses being 39 μM and 230 μM, respectively.

Blocking of the thiol group by acetylation, benzoylation or oxidation abolished the effectiveness of CoASH (table 1). As to the specificity, table 2 indicates that other thiol compounds, such as dithiothreitol, 2-mercaptoethanol or glutathione had little or no effect when tested at up to 5 mM and 2.5 mM, respectively. Moreover, the thiol reagents studied did not influence the effect of CoASH when added together to the reaction mixture (table 2).

Table 1

Essential role of free sulfhydryl group of coenzyme A

Expt.	Addition	PDH activity (mU/assay) at			ΔPDH inactivation (mU/assay) by effector added
		0 min	10 min	Δ10 min	
I	Buffer	36	17	19	–
	Acetyl-CoA (20 μM)	36	17	19	0
	CoASH (20 μM)	35	9	26	7
	Dephospho-CoASH (20 μM)	37	9	28	9
II	Buffer	90	57	33	–
	Benzoyl-CoA (29 μM)	90	57	33	0
	CoAS-SCoA (39 μM)	93	57	36	3
	CoASH (35 μM)	93	14	79	46

The conditions of PDH inactivation were similar to those in fig.2

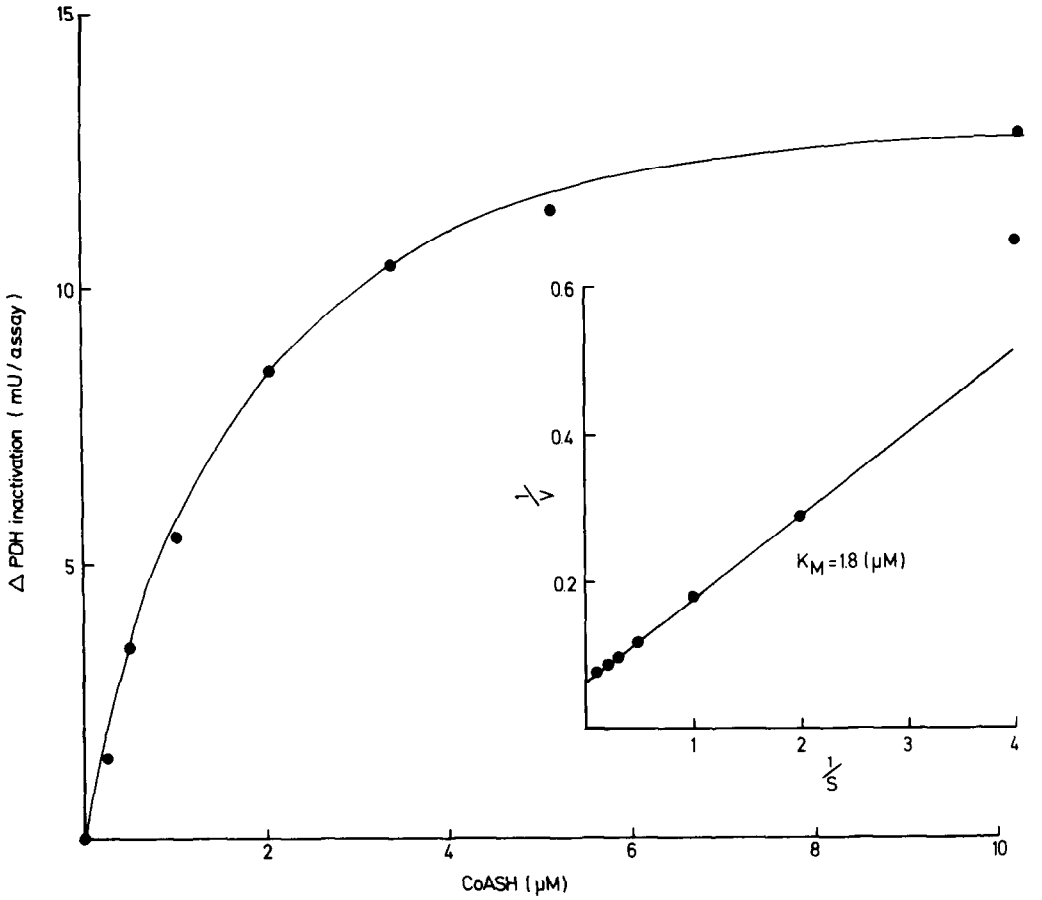

Fig.3. Stimulation of PDH inactivation as a function of CoASH concentration. The reaction mixture consisting of 20 μl dialyzed PDH in 50 mM histidine-HCl buffer (pH 6.9), 5 μl 1 mM ATP, 5 μl 2 mM $MgCl_2$ and 5 μl CoASH solution to yield the final concentrations indicated on the abscissa was incubated at 25°C for 0 and 15 min before 10 μl aliquots were analyzed for PDH activity. In the absence of added CoASH PDH activity at 0 and 15 min amounted to 29.2 and 15.7 mU/assay, respectively. The increase by added CoASH in PDH inactivation is indicated on the ordinate as ΔPDH inactivation.

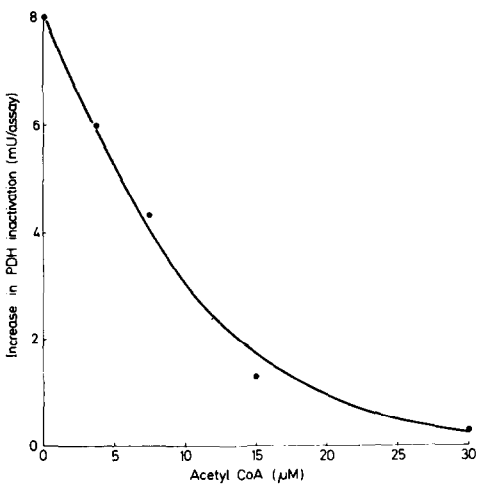

Fig.4. Release of CoASH stimulation of PDH inactivation by acetyl-CoA. A reaction mixture containing 20 μl dialyzed PDH in 50 mM histidine–HCl buffer (pH 6.9), 5 μl 2 mM $MgCl_2$, 5 μl 0.5 mM ATP and 5 μl 0.24 mM CoASH in the above buffer or buffer only was incubated at 25°C in the presence of 5 μl acetyl-CoA solution to yield the final concentrations indicated on the abscissa. At 0 and 10 min incubation 10 μl aliquots of the reaction mixture were assayed for PDH activity. In the absence of added CoASH and acetyl-CoA PDH activity amounted to 29.0 and 14.3 mU/assay at 0 and 10 min incubation, respectively. The increase by CoASH in PDH inactivation is given on the ordinate.

Table 2

Effect of CoASH and other sulfhydryl compounds on the rate of PDH inactivation

Additions	mM	PDH activity (mU/assay) at 0 min	10 min	Δ10 min	ΔPDH inactivation (mU/assay)
Control		100	45	55	–
2-Mercapto-	0.25	100	40	60	5
ethanol	5.0	100	39	61	6
Dithiothreitol	0.25	108	43	65	10
	5.0	102	40	62	7
Glutathione	0.125	98	47	51	4
	0.25	100	45	55	0
Coenzyme A	0.025	100	5	95	40
Coenzyme A 2-mercapto- ethanol	0.025+ 5.0	103	5	98	43
Coenzyme A dithiothreitol	0.025+ 5.0	105	5	100	45

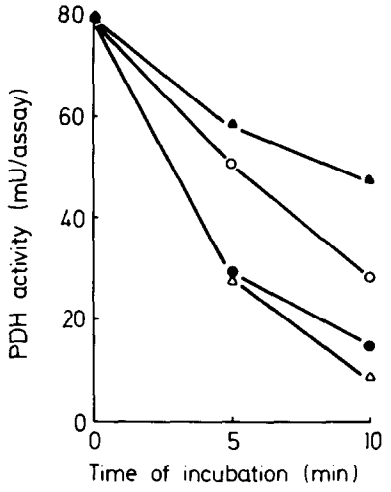

Fig.5. Lack of the acetyl-CoA effect after preincubation of PDH with CoASH. A sample of 30 μl dialyzed PDH in 20 mM potassium phosphate buffer (pH 7.0) was preincubated at 25°C for 5 min with 5 μl 0.33 mM CoASH in the same buffer (•) or buffer only (○, ▲, △) before the inactivation of the complex was initiated with 5 μl 1 mM ATP and 5 μl 2 mM $MgCl_2$ in the presence of the following addition(s): (▲) 10 μl of the above phosphate buffer; (△) 5 μl 0.33 mM CoASH + 5 μl phosphate buffer; (○) 5 μl 0.33 mM CoASH + 5 μl 0.33 mM acetyl-CoA; (•) 5 μl 0.33 mM acetyl-CoA + 5 μl phosphate buffer.

Fig.4 shows that acetyl-CoA was able to diminish the stimulation by CoASH of PDH inactivation. At 30 μM acetyl-CoA the effect of a saturating concentration (30 μM) of CoASH was almost completely abolished (fig.4). In the presence of 15 μM acetyl-CoA the [CoASH] yielding 50% effect was found to be increased from 1.8 to 120 μM. (After alkaline hydrolysis the acetyl-CoA sample was no longer antagonistic but rather increased PDH inactivation according to its expected CoASH content.) The acetyl-CoA, however, displayed its effect only if it was added simultaneously with CoASH to the reaction mixture. It was considerably less effective on PDH preparations that had been preincubated with CoASH (fig.5).

In addition to acetyl-CoA, pyruvate at 5 μM lowered the effectiveness of CoASH by 50%. ADP (0.5 mM), however, and inorganic pyrophosphate (20 μM), known inhibitors of PDH kinase [9–14], did not influence the effect of CoASH.

4. DISCUSSION

Our results are compatible with the view that PDH preparations contain a certain amount of CoASH which significantly affects the rate of PDH phosphorylation. Furthermore, it is reasonable to assume that this endogenous CoASH is (at least in part) removed during the purification procedure, especially by the dialysis step. In our experience

neither the duration nor the volume of the dialysis was the critical factor but rather the contact with the dialysis bag itself. In fact, repeated contacts for only a few minutes of a PDH preparation with the dialysis bag in the absence of any 'external' fluid resulted in an even more sluggish rate of inactivation than that reported herein. This influence of the bag was observed independent of extensive washings with various buffers in the absence and presence of adenine nucleotides, inorganic pyrophosphate, EDTA or dithiothreitol, but was no longer observed after pre-treatment with 1 mM CoASH (0.1 mM being insufficient). Thus one might presume that the bag had a remarkable binding capacity for CoASH and was able to detach it from PDH. This view is in line with our observation that by the addition of 10–30 μM CoASH to the PDH sample prior to dialysis a loss in PDH kinase activity could be prevented.

The finding of a stimulatory involvement of CoASH in PDH phosphorylation is of potential significance for the interpretation of a recent study on the regulation of PDH-kinase activity by thiol. Our preliminary experiments on the incorporation of [^{32}P]CoASH [15] into the component enzymes of the PDH-complex indicate the transacetylase core as the main acceptor. Although we could so far not distinguish between CoASH binding by the transacetylase itself and the kinase attached to it, it is tempting to speculate that the thiol group necessary for unimpaired PDH kinase function [1] may be that of CoASH.

These results are hard to reconcile with the current view on the regulation of the kinase. Thus, contrary to the present results, the rate of PDH inactivation by PDH kinase has been shown to depend on the proportion of acetyl-CoA:CoASH, a high ratio being favourable [16,17]. The reason for the difference between these and our results is unclear at present.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, FRG.

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